Identification of Residues Involved in a Conformational Change Accompanying Substitutions for Glutamate-43 in Staphylococcal Nuclease†

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ABSTRACT: A recent paper from our laboratories [Hibler, D. W., Stolowich, N. J., Reynolds, M. A., Gerlt, J. A., Wilde, J. A., & Bolton, P. H. (1987) *Biochemistry 26*, 6278] described the generation of site-directed substitutions for the putative general base Glu-43 in the active site of Staphylococcal nuclease (SNase) and the use of ^{1}H NMR spectroscopy to characterize the effect of the substitutions on the conformations of the mutant proteins. The replacements for Glu-43 (Asp, Gln, Asn, Ser, and Ala) both decreased the catalytic efficiency and changed the one- and two-dimensional NMR spectral properties of the mutant enzymes. We have prepared and studied the NMR spectral properties of several samples of deuteriated wild-type SNase that allow sequence-specific resonance assignments for several aromatic and aliphatic amino acid side chains that experience changes both in normal one-dimensional spectra and in two-dimensional NOESY spectra. Due to severe spectral congestion of resonances in the one- and two-dimensional spectra of protiated SNase, the assignments would have been difficult, if not impossible, to obtain without deuteriation of selected amino acids. The spectra we have obtained demonstrate that changes in NOE intensities involve a valine residue that is spatially adjacent to *two* phenylalanine residues; given the X-ray structure for SNase [Cotton, F. A., Hazen, E. E., & Legg, M. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2551], these residues must be Val-74, Phe-34, and Phe-76. In addition, a leucine residue experiencing changes in NOE intensities spatially adjacent to Val-74 and Phe-34 can be assigned to Leu-25. We have also obtained spectra of mutant enzymes with amino acid substitutions of these and spatially adjacent residues that reveal significant changes in the upfield-shifted methyl regions, thereby supporting our assignments. These assignments demonstrate that substitutions at residue 43 in the active site produce a conformational change that extends into a region of the protein molecule that is between 15 (Leu-25) and 30 Å (Phe-76) from the position of the substitution. This now unequivocal conclusion regarding the extent and magnitude of conformational changes in mutants of SNase in which active-site residues have been replaced by site-directed mutagenesis emphasizes the caution that must be exercised in quantitatively interpreting the effects of (specific or nonspecific) amino acid substitutions on the catalytic efficiency of mutated enzymes.

Techniques are now available for the systematic alteration of the primary structure of an enzyme so that structure–function relationships in catalysis might be investigated (Ackers & Smith, 1985; Leatherbarrow & Fersht, 1986; Gerlt, 1987; Knowles, 1987; Shaw, 1987). We have altered the amino acid at position 43 in Staphylococcal nuclease (SNase), which in the wild-type enzyme is the putative glutamate general base involved in the hydrolysis reaction (Hibler et al., 1987). Our previous studies involved five substitutions for this residue (E43D, E43Q, E43N, E43S, and E43A), and the structural characterization by ^{1}H NMR spectroscopy focused on two substitutions: the homologous aspartate substitution (E43D), which decreased V_{max}/K_m 1400-fold, and the uncharged serine substitution (E43S), which decreased the catalytic efficiency 5000-fold. Both of these substitutions were observed to alter the NMR chemical shifts of aromatic and upfield-shifted aliphatic protons as well as the nuclear Overhauser effects (NOEs) between protons associated with these spectral regions. The increases and decreases of NOEs are in some cases 2-fold, which is consistent with changes in certain interresidue separations on the order of 0.5 Å. Since the catalytic efficiency of any enzymatic reaction is likely to be sensitive to small changes in conformation, an understanding of the observed spectral changes in terms of the location and magnitude of the responsible conformational change(s) is required before structure–function relationships in catalysis can be either qualitatively or quantitatively formulated.

Our previous studies described NMR spectral data that were consistent with changes in interresidue separations between phenylalanine, tyrosine, and valine residues and suggested that these changes involved Phe-34 and/or Phe-76, Val-74, and Tyr-27; all of these residues are removed by at least 15 Å from the site of the substitutions at residue 43. However, the data then available were insufficient to obtain site-specific assignments of these resonances since the resonances associated with the three phenylalanine residues in SNase are partially coincident and the resonances in the upfield-shifted methyl region could not be assigned to specific amino acid residues or even amino acid type. Rather than resorting to or waiting for total chemical shift assignments of SNase, we have investigated deuteriated samples and specific mutants, the results of which together with the X-ray structure of SNase (Cotton et al.,

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1979) permit unequivocal chemical shift assignments of some of the protons involved in the conformational change. Our results also illustrate the power of deuteriation in simplifying the one- and two-dimensional spectra of proteins of moderate size such as SNase and suggest that specific mutants generated by site-directed mutagenesis might be generally useful in making residue-specific chemical shift assignments. In this paper we summarize ^{1}H NMR spectral data obtained on both deuteriated samples and mutants of SNase that allow the identification of a conformational change in E43D and E43S to residues remote (between 15 and 30 Å) from the position of the substitution in the active site.

Materials and Methods

The plasmid pNJS was previously described (Hibler et al., 1987). In this plasmid the gene for an N-terminal-modified SNase is under the control of the bacteriophage λ P_R promoter; the plasmid also carries the gene for the cI857 temperature-sensitive mutation of the λ repressor so a transformed host cell can be induced for production of SNase by an increase in the temperature of the growth medium from 30 to 42 °C. This plasmid is routinely propagated in *Escherichia coli* strain N4830; this strain is auxotrophic for isoleucine, histidine, and valine and also carries the gene for the cI857 mutation of the λ repressor in its chromosome.

The plasmids containing the F34Y, F76V, H124L, and H124R mutations were obtained from Professor David Shortle, Johns Hopkins Unversity School of Medicine (Shortle & Lin, 1985). In these plasmids the genes for hybrid preenzymes of the mutant versions of SNase are under the control of the *pho*A promoter so a transformed *E. coli* host cell can be induced for production of SNase by deprivation of inorganic phosphate.

The plasmids containing the Y27F, Y54F, Y85F, Y113F, and Y115F substitutions in pONF1 (Takahara et al., 1985) were constructed by Dr. Mark A. Reynolds and Lynn Harpold using the double-primer method described by Zoller and Smith (1984); the details of these mutageneses parallel those described earlier for E43D and E43S (Hibler et al., 1987) and will be described in detail in a subsequent publication.

[*ring*-$^2H_5$]-L-Phenylalanine, [*indole*-$^2H_5$]-L-tryptophan, [$^2H_8$]-L-valine, and [$^2H_{10}$]-L-leucine were purchased from MSD Isotopes. [3(*S*)-C^2H_3]-L-Valine was the generous gift of Dr. John Aberhart, Worcester Foundation for Experimental Biology, Shrewsbury, MA.

[*ring*-2,6-$^2H_2$]-DL-Phenylalanine and [3,4,5,α,β_2-$^2H_6$]-DL-phenylalanine ([*ring*-3,4,5-$^2H_3$]-DL-phenylalanine) were prepared by Raney nickel catalyzed exchange of [*ring*-$^2H_5$]-L-phenylalanine with H_2O and of L-phenylalanine with ^2H_2O, respectively (Woodworth & Dobson, 1979).

Isolation of Protiated and Deuteriated Enzymes. The isolation of wild-type SNase from N4830 transformed with pNJS and the procedure for the biosynthetic incorporation of aromatic amino acids into this enzyme were described previously (Hibler et al., 1987).

The incorporation of perdeuteriated leucine or valine into the SNase encoded by pNJS was accomplished by procedure analogous to that described for the incorporation of aromatic amino acids except that in the case of valine the inoculum also contained the labeled amino acid at a concentration of 200 mg/L since the host is a valine auxotroph. The incorporation of the chirally trideuteriated valine was similarly accomplished but on a smaller scale (250 mL) to conserve the labeled amino acid; for the preparation of this enzyme the isotopically labeled valine was present in both the inoculum and the final culture medium at a concentration of 25 mg/L.

^{1}H NMR Studies. Samples of protiated and deuteriated enzymes were prepared for ^{1}H NMR spectroscopy as previously described (Hibler et al., 1987). All ^{1}H NMR spectra were obtained at 30 °C on a Varian XL-400 NMR spectrometer. The two-dimensional NOESY spectra were obtained with a 150-ms mixing time.

Results and Discussion

Our previous paper (Hibler et al., 1987) described the preparation of samples of SNase containing deuteriated aromatic amino acids and the use of these samples to conclude that some of the upfield-shifted methyl groups that experience changes in chemical shift and magnitudes of NOEs to aromatic protons are spatially adjacent to a phenylalanine residue or residues and perhaps the protons meta to the hydroxyl group in a tyrosine residue. The evidence for this conclusion was based upon the diminished intensities of specific NOEs in a sample containing [*ring*-$^2H_5$]-L-phenylalanine and the complete absence of NOEs in a sample additionally containing [2,6-$^2H_2$]-L-tyrosine. Examination of the X-ray structure for the wild-type SNase (Cotton et al., 1979) suggested that the only residues satisfying the constraints of an aliphatic methyl group proximal to both phenylalanine and tyrosine residues are Val-74, Phe-34, and/or Phe-76, and Tyr-27. However, the NOE contribution by the tyrosine residue was weak, and we felt that further evidence was necessary for the unambiguous localization of the conformational change to this region that is between 15 and 30 Å removed from the position of the substitutions for Glu-43.

We have now confirmed this suggestion by the preparation and NMR spectral analysis of several additional deuteriated samples of wild-type SNase encoded by the gene in the plasmid pNJS. The one- and two-dimensional spectra obtained on these samples permit assignment of the NOEs between the upfield-shifted methyl groups of Leu-25 and Val-74 to each of the protons on the aromatic rings of Phe-34 and Phe-76. These assignments are also consistent with the upfield-shifted methyl regions of the spectra of several mutants containing a single substitution in this structural region.

Deuteriated Samples. We have biosynthetically prepared six additional samples of wild-type SNase labeled with the following deuteriated amino acids: d_{10}-L, [$^2H_{10}$]-L-Leu; d_8-V, [$^2H_8$]-L-Val; d_3-V, [3(*S*)-C^2H_3]-L-Val; h_5-F, [*ring*-$^2H_4$]-L-Tyr and [*indole*-$^2H_5$]-L-Trp; h_2-F, [*ring*-3,4,5-$^2H_3$]-L-Phe, [*ring*-$^2H_4$]-L-Tyr, and [*indole*-$^2H_5$]-L-Trp; and h_3-F, [*ring*-2,6-$^2H_2$]-L-Phe, [*ring*-$^2H_4$]-L-Tyr, and [*indole*-$^2H_5$]-L-Trp.

One-Dimensional Spectra of Deuteriated Samples. The upfield-shifted methyl group region of spectra of d_{10}-L, d_8-V, and d_3-V are compared with protiated wild-type SNase in Figure 1. Comparison of these spectra reveals that the upfield-shifted methyl groups with resonances at −0.03 and 0.43 ppm that were previously associated with an isopropyl group in a valine or leucine residue are, in fact, associated with a valine residue; these resonances were observed to shift upfield slightly in E43D and E43S and experience diminished NOEs to phenylalanine protons at 7.03 ppm in the mutant enzymes (Hibler et al., 1987). This comparison also shows the presence of two resonances associated with the methyl groups of a leucine residue or residues at 0.11 and 0.82 ppm; the NOESY spectrum of protiated SNase (not shown) shows that these two resonances are correlated and, therefore, are associated with the same leucine residue. These leucine protons are also observed to experience both changes in chemical shift and decreases in NOEs to the same phenylalanine protons at 7.03 ppm in the mutant enzymes. Additional resonances that can be assigned to valine and leucine are also apparent in this

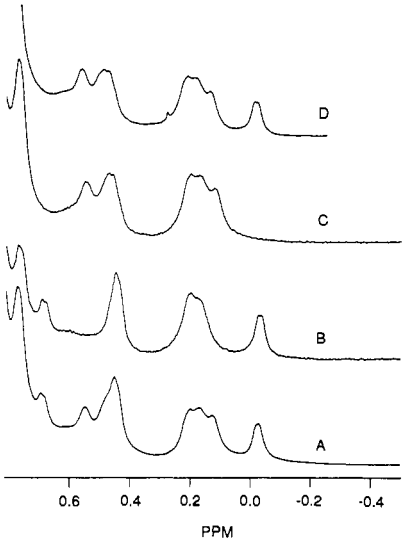

FIGURE 1: Comparison of the upfield-shifted methyl group regions of ^{1}H NMR spectra of protiated SNase (A), d_{10}-L (B), d_8-V (C), and d_3-V (D).

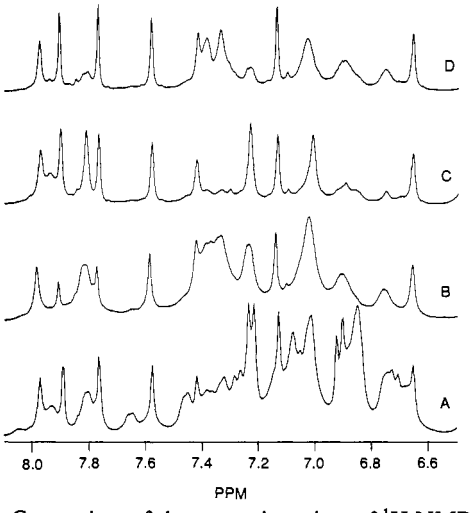

FIGURE 2: Comparison of the aromatic regions of ^{1}H NMR spectra of protiated SNase (A), h_5-F (B), h_2-F (C), and h_3-F (D).

spectral region. The resonances at 0.17 and 0.20 ppm are not associated with leucine or valine and, due to the apparent complexity in their vicinal coupling, are likely to be associated with an isoleucine residue or residues.

A small sample of [3(*S*)-C^2H_3]-L-valine was kindly made available to us by Dr. John Aberhart, Worcester Foundation for Experimental Biology. The biosynthetic incorporation of this material into wild-type SNase allows the assignment of the two methyl groups whose resonances appear at −0.03 and 0.43 ppm to the diastereotopic methyl groups of the upfield-shifted valine residue. Comparison of the upfield-shifted methyl regions of d_8-V and d_3-V in Figure 1 reveals that the valine methyl resonance at 0.43 ppm is missing in the spectrum of d_3-V, thereby allowing the assignment of this methyl group as the *pro-S* methyl group; the well-resolved, most upfield shifted resonance is, therefore, associated with the *pro-R* methyl group of the same valine residue.

The aromatic regions of the spectra of h_5-F, h_2-F, and h_3-F are compared with that of protiated wild-type SNase in Figure 2. The spectrum of h_5-F contains only those resonances associated with the 15 protons of the three phenylalanine and the 8 protons of the four histidine residues. Further spectral simplification is accomplished by deuteriation of the meta and para protons (h_2-F) or the ortho protons (h_3-F). The spectra

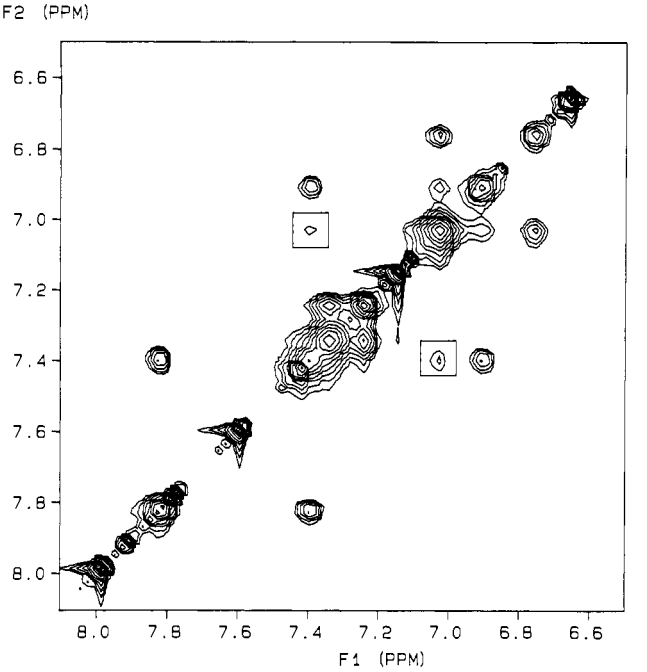

FIGURE 3: Subsection of the NOESY spectrum of h_5-F showing the correlations among the aromatic protons. The boxed correlation at (7.4, 7.03 ppm) is associated with an amide proton.

demonstrate that the resonances associated with the ortho protons of the three phenylalanine residues occur at 7.00, 7.25, and 7.80 ppm (spectrum 2C) and that the resonances associated with the meta and para protons occur at 6.75, 6.90, 7.0, 7.33, and 7.38 ppm (spectrum 2D); note that the expected number of resonances is observed for the ortho protons but that a coincidence of two resonances occurs for the meta and para protons.

Two-Dimensional Spectra of Deuteriated Samples: Aromatic–Aromatic Correlations. The subsection of a NOESY spectrum of h_5-F showing the correlations among the aromatic protons of the three phenylalanine residues is reproduced in Figure 3. This NOESY spectrum reveals the presence of four correlations, one of which at (7.4, 7.03 ppm) is associated with a correlation involving an amide proton. In principle, six intraresidue correlations from the phenylalanines could have been present, and, as detailed later, the three missing correlations are too close to the diagonal to be distinguished from the diagonal signals.

NOESY spectra of h_2-F and h_3-F allow the correlations shown in Figure 3 to be assigned to ortho, meta, and meta, para correlations. As shown in Figure 4, the NOESY spectrum of h_3-F reveals the presence of two meta, para correlations at (7.4, 6.90 ppm) and (7.03, 6.75 ppm) and the amide correlation at (7.4, 7.03 ppm). (The resolution of our NOESY spectra is insufficient to resolve the protons with resonances at 7.33 and 7.38 ppm, and in the following discussion these are designated 7.4 ppm.) Thus, only one ortho, meta correlaton is present in the spectrum of the protiated SNase, and two ortho, meta correlations must be located close to the diagonal.

Aliphatic–Aromatic Correlations. The subsection of a NOESY spectrum of h_5-F showing the correlations among the phenylalanine and histidine protons and the upfield aliphatic protons is reproduced in Figure 5. The correlations present in this spectral region can be best analyzed if vertical slices through the resonances associated with methyl groups of both valine and leucine residues are examined since these reveal resolved correlations to the phenylalanine protons.

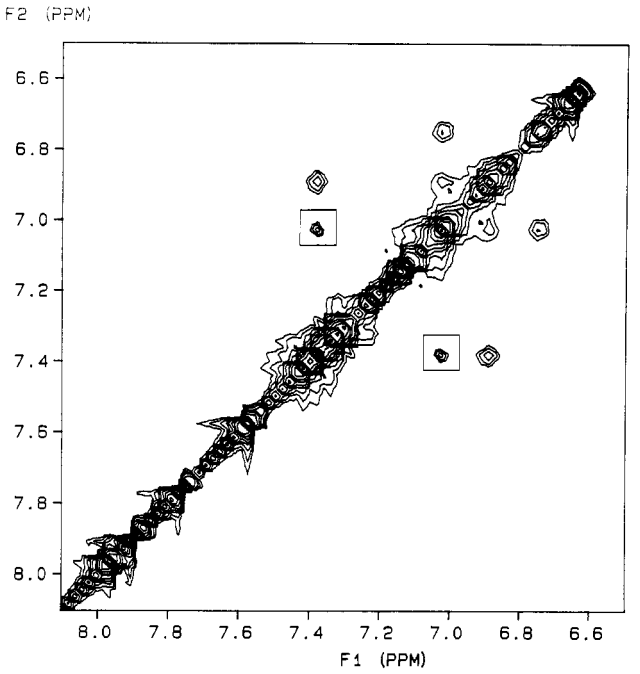

FIGURE 4: Subsection of the NOESY spectrum of h_3-F showing the correlations among the aromatic protons. The boxed correlation at (7.4, 7.03 ppm) is associated with an amide proton.

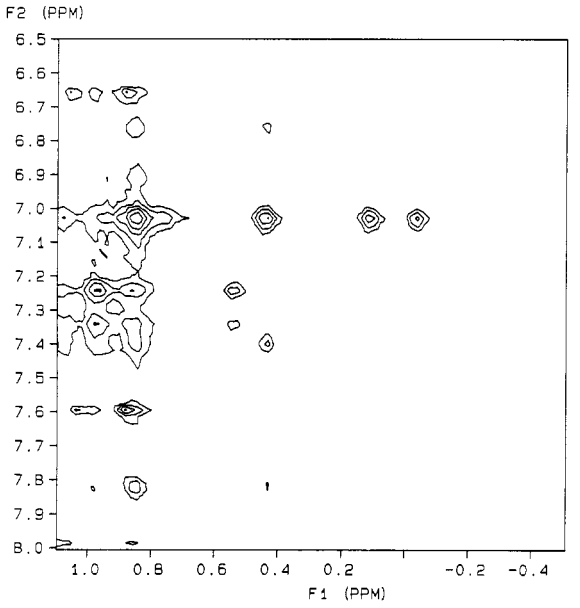

FIGURE 5: Subsection of the NOESY spectrum of h_5-F showing the correlations between the aromatic and upfield methyl protons.

Figures 6–8 show the vertical slices correlating the *pro-R* valine methyl group at −0.03 ppm, the *pro-S* valine methyl group at 0.43 ppm, and the upfield leucine methyl groups at 0.11 ppm; three slices are shown for each methyl group, namely, for h_5-F (spectra A), h_3-F (spectra B), and h_2-F (spectra C).

The three slices obtained from the spectrum for h_2-F are particularly noteworthy. Both valine methyl groups have correlations to the ortho protons of *two* phenylalanine residues (7.00 and 7.80 ppm); the leucine methyl group has a correlation to the ortho protons of *one* of the same phenylalanine residues (7.00 ppm). This observation clearly demonstrates that the valine residue is spatially adjacent to two phenylalanine residues, and *the only phenylalanine and valine residues that structurally satisfy this constraint* (Cotton et al., 1979) *are Phe-34, Phe-76, and Val-74.* The residue assignments for the resonances associated with each phenylalanine residue and the leucine residue will be discussed later.

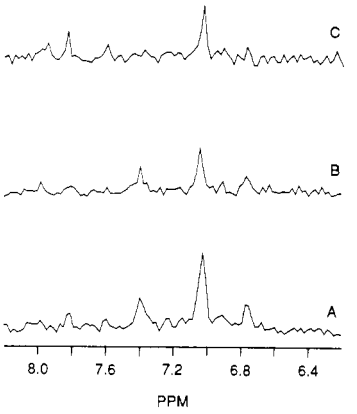

FIGURE 6: Vertical slices from NOESY spectra showing the aromatic correlations involving the *pro-R* valine methyl group at −0.03 ppm. Spectrum A is taken from a spectrum of h_5-F, spectrum B is taken from a spectrum of h_3-F, and spectrum C is taken from a spectrum of h_2-F.

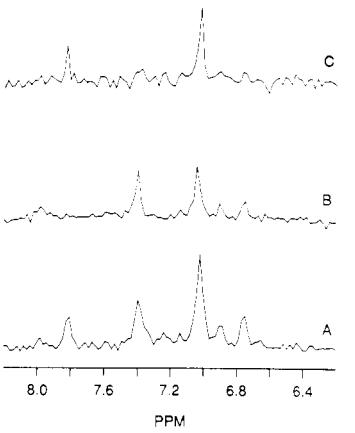

FIGURE 7: Vertical slices from NOESY spectra showing the aromatic correlations involving the *pro-S* valine methyl group at 0.43 ppm. Spectrum A is taken from a spectrum of h_5-F, spectrum B is taken from a spectrum of h_3-F, and spectrum C is taken from a spectrum of h_2-F.

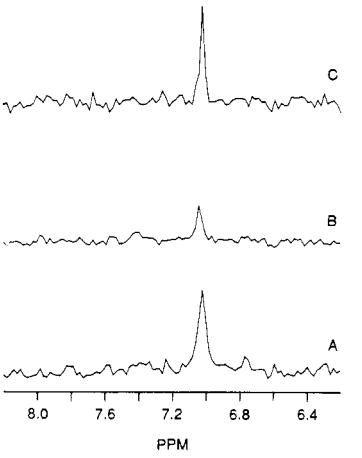

FIGURE 8: Vertical slices from NOESY spectra showing the aromatic correlations involving the upfield leucine methyl group at 0.11 ppm. Spectrum A is taken from a spectrum of h_5-F, spectrum B is taken from a spectrum of h_3-F, and spectrum C is taken from a spectrum of h_2-F.

The slice for the *pro-S* methyl group of Val-74 obtained from the spectrum of h_3-F clearly reveals the chemical shifts of the meta and para protons for Phe-34 and -76: 7.4, 7.03, 6.90, and 6.75 ppm. These four chemical shifts are the

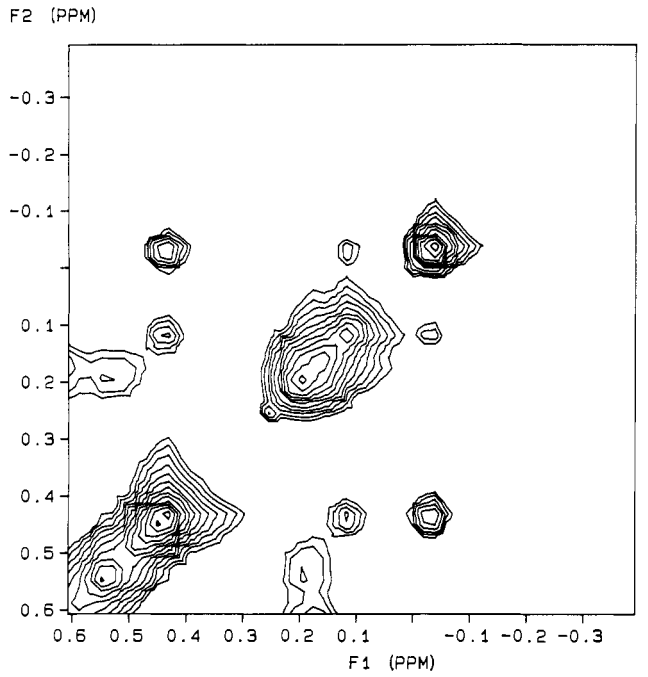

FIGURE 9: Subsection of the NOESY spectrum of h_5-F showing the correlations among the upfield-shifted methyl group protons.

chemical shifts observed in the meta, para correlations. Referring to Figure 2, the intensities of the resonances at 6.90 and 6.75 ppm and the apparent independence of the line widths of these resonances on deuteriation of the ortho positions suggest that the resonances at 7.4 and 7.03 ppm are associated with meta protons and those at 6.90 and 6.75 ppm are associated with para protons; these assignments are in accord with the observed correlations in Figures 3 and 4. The slice for the methyl group of the leucine residue from the spectrum of h_3-F reveals the presence of a strong NOE correlation only to the meta proton at 7.03 ppm with no apparent correlation to the para protons at 6.90 and 6.75 ppm (although the spectrum of h_5-F appears to show a weak NOE to the para proton at 6.75 ppm).

Taken together, the observed correlations are consistent with one of the phenylalanine residues spatially adjacent to Val-74 having the resonances for its ortho, meta, and para protons at 7.80, 7.4, and 6.90 ppm, respectively, and the second phenylalanine residue having the resonances for its ortho, meta, and para protons at 7.00, 7.03, and 6.75 ppm, respectively (note that this phenylalanine residue has nearly coincident resonances for its ortho and meta protons). By difference, the resonances for the ortho, meta, and para protons for the phenylalanine residue spatially distal to Val-74 (necessarily Phe-61 given the X-ray structure) can be assigned to those at 7.25, 7.4, and 7.4 ppm, respectively. These groupings for the phenylalanine residues spatially proximal to Val-74 are consistent with the observation of weak interresidue phenylalanine NOE correlations at (6.75, 6.90 ppm) and [6.90, 7.00 (or 7.03) ppm] since the X-ray structure reveals that Phe-34 and Phe-76 are spatially proximal. However, additional NOE data to be described in the next section are necessary for unequivocal assignments for these phenylalanine residues.

Aliphatic–Aliphatic Correlations. The residue assignments of the upfield-shifted leucine methyl groups and of the phenylalanine residues that are spatially proximal to Val-74 cannot be made without information about the spatial proximity of Val-74 to the leucine residue. Examination of the X-ray structure for wild-type SNase (Cotton et al., 1979) reveals that Leu-25 is spatially adjacent to Phe-34 and Val-74; similarly,

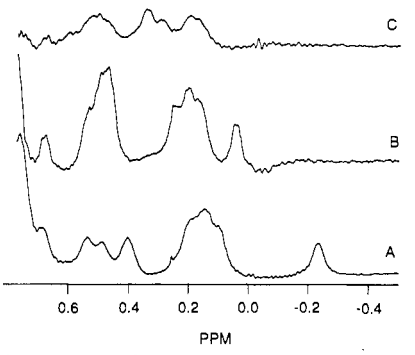

FIGURE 10: Comparison of the upfield-shifted methyl group regions of ^{1}H NMR spectra of Y27F (A), F34Y (B), and F76V (C).

Leu-7 is spatially adjacent to Phe-76 but not to Val-74.

Figure 9 reproduces a contour map that shows the NOE correlations in the upfield-shifted methyl group region. Both the *pro-R* (–0.03 ppm) and *pro-S* (0.43 ppm) methyl groups of Val-74 are spatially adjacent to the upfield methyl group (0.11 ppm) of the leucine residue that has correlations to only one of the phenylalanine residues adjacent to Val-74. Thus, the leucine residue can be assigned to Leu-25. Furthermore, the phenylalanine residue involving an ortho proton at 7.00 ppm can be assigned to Phe-34, and the phenylalanine residue involving an ortho proton at 7.80 ppm can be assigned to Phe-76.

Upfield-Shifted Methyl Group Regions of Mutant Enzymes. The residue assignments made in the previous sections are based upon the high-resolution X-ray structure of SNase (Cotton et al., 1979). We have also recorded the one-dimensional ^{1}H NMR spectra of mutants of SNase in which residues either including or in the structural vicinity of Phe-34, Val-74, and Phe-76 have been substituted (as deduced from the X-ray structure). The upfield-shifted regions of the spectra for the Y27F, F34Y, and F76V mutants are shown in Figure 10. These spectra provide independent support for the assignment of Val-74 to the most upfield shifted resonance in the spectrum of SNase; the spectra of all three mutant enzymes show significantly perturbed chemical shifts in this spectral region, suggesting that these substitutions alter the ring current effects that are responsible for the upfield chemical shifts of the methyl groups of Val-74 (as well as several other methyl groups). The spectra of a number of additional aromatic substitutions elsewhere in the structure of the protein have been determined, including Y54F, Y85F, Y113F, Y115F, H124R, and H124L, and none of these show significantly perturbed chemical shifts in the upfield-shifted methyl region (data not shown).

CONCLUSIONS

With information provided by both deuteriated samples of SNase and the high-resolution X-ray structure (Cotton et al., 1979), we have accomplished consistent chemical shift assignments for the aromatic protons of the three phenylalanine residues in wild-type SNase as well as the methyl groups of Val-74 and Leu-25 residues that are spatially proximal to two of these phenylalanine residues, i.e., Phe-34 and Phe-76. It is pertinent to note that only Phe-61 is located within an α-helix, and, as such, the resonances associated with its protons might be expeditiously assigned by more traditional techniques (Wüthrich, 1986; Englander & Wand, 1987). We do not believe that our use of the available X-ray structure of wild-type SNase in making these assignments is incorrect, given the improbability that a protein structure containing only three phenylalanine residues would randomly have a single valine

residue spatially adjacent to two phenylalanine residues. Rather, we believe that in the case of a "large" protein such as SNase the X-ray data can be used *qualitatively* to assist in ^{1}H NMR chemical shift assignments unless the available structural and NMR data are clearly contradictory. In addition, deuteriation of additional aliphatic residues as well as interresidue "NOE walking" within the hydrophobic core of SNase guided by its X-ray structure might allow additional chemical shift assignments to be accomplished and the extent of the conformational change induced by the active-site substitutions to be completely assessed.

We can now be confident that the substitutions we have generated for Glu-43 in the active site, including E43D and E43S, are accompanied by a conformational change that includes the residues we have assigned. Leu-25 is the most proximal of these resiudes to residue 43 at a distance of about 15 Å; Phe-76 is the most distal at a distance of about 30 Å. Our two-dimensional spectra of E43D and E43S show that the magnitudes of the interresidue NOE correlations involving these phenylalanine, valine, and leucine residues are significantly different from those observed in wild-type SNase. While the analysis of our two-dimensional data for wild-type and mutant enzymes does not yet allow a conclusion as to whether a few or all of these residues experience changes in position in the mutant enzymes, the conclusion is clear that the substitutions introduced by site-directed mutagenesis do cause changes in secondary and tertiary structure that can be presumed to influence the catalytic efficiencies of the mutant enzymes. With the chemical shift assignments described in this paper the conformational changes occurring in the mutant proteins can now be analyzed in greater detail by quantitation of the changes in the magnitudes of the NOE correlations we have described.

Finally, to the best of our knowledge, the assignments reported in this paper provide the first irrefutable evidence that site-directed substitutions of active-site residues can cause extensive conformational changes within enzymes in solution. In the terminology proposed by Fersht et al. (1987), our E43D and E43S mutations are structurally disruptive substitutions and deletions, respectively, although the E43D mutation naively might have been considered to be a homologous and, therefore, a structurally conservative substitution. The fact that a homologous substitution can, in fact, be structurally disruptive confirms our previous caution (Hibler et al., 1987) that mechanistic (i.e., quantitative) interpretations of the properties of site-directed substitutions of active-site residues [i.e., Serpersu et al. (1987)] in the absence of assessment of the effect of the substitution on the conformation of the mutant protein *in solution* may well be prone to serious errors of interpretation. Our studies of SNase have provided substantive data that demonstrate that at least in this protein extreme care must be exercised in the quantitative interpretation of the kinetic phenotypes of site-directed substitutions; the burden of assessing whether such care must be exercised in other systems must be assumed by those generating and characterizing such mutants.

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